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TITLE: Gamma Synuclein Promotes a Metastatic Phenotype in Breast
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Transduction Activity

PRINCIPAL INVESTIGATOR: Andrew K. Godwin, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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13. Abstract (Maximum 200 words) <p>Synucleins (a, b, g, synuclein) are a family of small, highly conserved proteins expressed predominantly in neurons. While a-synuclein is implicated in neurodegenerative diseases, g-synuclein is expressed in the majority (> 85%) of late-stage breast and ovarian carcinomas and is not expressed in normal mammary and ovarian epithelium. In spite of their significance, the normal and pathological roles of synucleins are not fully understood. To address the biological function of g-synuclein and its role in the malignancy of breast and ovarian cancer, we ectopically over-expressed g-synuclein in several cancer cell lines. Recently we found that g-synuclein is associated with two major mitogen-activated kinases (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1). Over-expression of g-synuclein lead to constitutive activation of ERK1/2, and down-regulation of JNK1 in response to stress (UV, sodium arsenate, and heat shock). In this study, we further characterized the effects of g-synuclein on paclitaxel, a commonly used chemotherapeutic drug, and nitric oxide induced apoptosis. We found that g-synuclein over-expressing cells were more resistant (4- to 5-fold) to paclitaxel or nitric oxide as compared to the parental cells. This resistance to paclitaxel could be partially restored when ERK activity was inhibited using U0126, a MEK1/2 inhibitor. In addition, activation of the mitochondrial apoptotic pathway (JNK and/or caspase 3 activation) by paclitaxel and nitric oxide was blocked by ectopic expression of g-synuclein. Collectively, these data indicate that g-synuclein may be involved in the pathogenesis of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain anti-cancer drugs. Because of its high frequency of expression in late-stage breast and ovarian cancers, g-synuclein may be a promising target for cancer therapy.</p>				
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Table of Contents

Front Cover	1
Standard Form 298	2
Table of Contents	3
Introduction	4
Body	4
Conclusions	17
References	18
Appendices	20

INTRODUCTION:

The synucleins (α , β , γ , synoretin) are a family of highly conserved small proteins that are normally expressed predominantly in neurons. Little is known about the normal functions of synucleins in physiological conditions. Of the synucleins, α -synuclein is the best characterized because of its potential significance in neurodegenerative diseases including Parkinson's Disease. Recently we and others have found that γ -synuclein is dramatically up-regulated in the vast majority of late-stage breast (70%) and ovarian (> 85%) cancers and that γ -synuclein over-expression can enhance tumorigenicity (Bruening et al., 2000; Ji et al., 1997; Liu et al., 2000). We also observed that expression of γ -synuclein induces a phenotype similar to that induced by activation of RhoA/Rac/CDC42, altering the appearance of focal adhesions and stress fibers, and enhancing motility and invasion in ovarian cancer cells. Recent studies by Ji and colleagues have also shown that when γ -synuclein is overexpressed in a breast tumor derived cell line, the cells experience a dramatic augmentation in their capacity to metastasize *in vivo* (Jia et al., 1999). Based on these known data, we hypothesized that γ -synuclein may be a proto-oncogene, and that the abnormal expression of this protein (i.e., oncogenic form) in breast and ovarian tumors may contribute to the metastatic spread and high morbidity associated with advance stages of these diseases. To address this hypothesis, we first ectopically over-expressed γ -synuclein in several cancer cell lines and the effects of γ -synuclein on the phenotypes of these cells were characterized. The mechanisms underlying γ -synuclein induced cell phenotype changes were investigated by biochemical and cellular assays. We found that γ -synuclein is associated with two major mitogen-activated kinases (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1). Over-expression of γ -synuclein may enhance cell motility by activating Rho/Rac/Cdc42 and ERK pathways. JNK activation induced by stress (UV, arsenate, and heat shock) and chemotherapy drugs (Taxol, vinblastine) can also be down-regulated by γ -synuclein. Finally, we found that γ -synuclein can protect chemotherapy drug-induced cell death by modulating the ERK cell survival pathway and the JNK-mitochondria-Caspase9/3 pathway.

BODY:

γ -Synuclein interacts with ERK and JNK MAP kinases in cancer cells - α -Synuclein has recently been reported to bind directly to the ERK2 kinase (Iwata et al., 2001b). Therefore, we evaluated whether γ -synuclein could also interact with the ERK kinases as well as other MAPKs. By co-immunoprecipitation

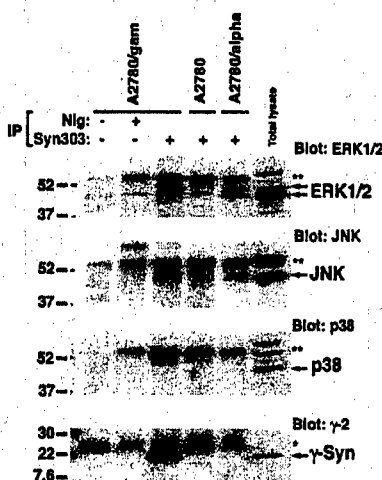


Fig. 1. Interaction between γ - and α -synucleins with ERK1/2 and JNK. Cell lysate from A2780, A2780/gam or A2780/alpha were immunoprecipitated with Syn303, Nig (normal IgG) or irrelevant antibodies (not shown here). The proteins in the immunoprecipitates were identified by immunoblotting with antibodies against ERK1/2, JNK1, p38, and γ -2 (a polyclonal antibody specific for γ -synuclein). Molecular mass standards (in kilodaltons) are indicated on the left. Non-specific bands around the IgG heavy (**) and light (*) chains are indicated by asterisks.

approaches, we were able to demonstrate a novel association of γ -synucleins with ERK1/2 and JNK1 kinase, but not with the p38 kinase (Fig. 1). We also confirmed that α -synuclein is associated with ERK1/2 as well with JNK1 (Fig. 1), which is consistent with the recently studies using neuro2a, a

neuronal cell line (Iwata et al., 2001a). These data indicate that γ - and α -synuclein can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.

Elevated activity of ERK in cells over-expressing γ -synuclein - We next evaluated whether these protein interactions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ -synuclein, the activated ERK1/2 was increased 2-3 fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (Fig. 2). In contrast, α - and β -synucleins

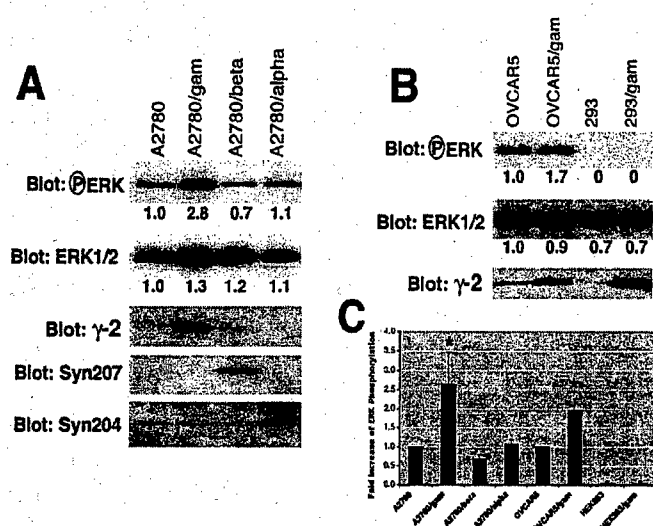


Fig. 2. Activation of ERK in cells over-expressing γ -synuclein. A, ERK1/2 activation is enhanced in γ -synuclein over-expressing A2780 cells. Whole cell lysates (40 μ g/lane) from A2780 cells (parent) or A2780 cells transfected with γ -, β -, or α -synuclein were separated by SDS-PAGE and blotted with appropriate antibodies. The levels of activated ERK or total ERK1/2 were determined using an anti-phospho-ERK specific antibody or ERK1/2 antibodies, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band (the integrated densitometry reading number of A2780 or OVCAR5 was assigned to an arbitrary unit 1.0, and the other readings were normalized thereafter). The synucleins were evaluated by blotting with specific antibodies, i.e., γ -2 for γ -synuclein, Syn207 for β -synuclein, and Syn204 for α -synuclein, respectively. B, activation of ERK by γ -synuclein in OVCAR5 cells but not HEK 293 cells. Whole cell lysate (40 μ g/lane) from parental or γ -synuclein over-expressing cells were separated and blotted as in A. Panels A and B are representative of at least three independent experiments with comparable results. C, fold increase of ERK activation. The data shown are the average \pm S.E. of three independent experiments. Phosphorylated ERK was normalized to the protein level of total ERK. The basal levels of ERK phosphorylation in the parental A2780 or OVCAR5 cells were set as 1.0. (*) Represents significant difference compared to the parental cells ($p < 0.05$).

for γ -synuclein mediated activation of ERK1/2. When cells over-expressing γ -synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (Fig. 3A). We further studied the relation of γ -synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ -synuclein and ERK1/2 was still present (Fig. 3B). These data indicate that γ -synuclein may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ -synuclein.

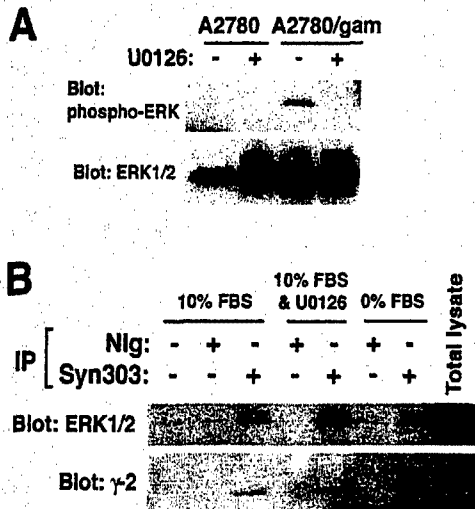


Fig. 3. Requirement of MEK1/2 for γ -synuclein enhanced ERK1/2 activation. **A**, A2780 and A2780/gam cells untreated or treated with the MEK1/2 inhibitor, U0126 (10 μ M), were lysed and 30 μ g of proteins were loaded into each lane. As in Fig. 2, anti-phospho-ERK1/2 specific antibody was used to detect activated ERK1/2; and the antibody against ERK1/2 was used to detect the protein level of total ERK1/2. **B**, the interaction between ERK and γ -synuclein is independent of the activation status of ERK1/2. A2780/gam cells in normal 10% FBS medium, 10% FBS medium with U0126 (10 μ M), or serum-free medium were lysed and immunoprecipitated with Syn303 or control IgG. The proteins in the immunoprecipitates were detected with the antibodies against ERK1/2 or against human γ -synuclein (γ -2). The autoradiogram shown is the representative of three independent experiments with comparable results.

Over-expression of γ -synuclein leads to increased cell motility- Recent studies by Jia and colleagues

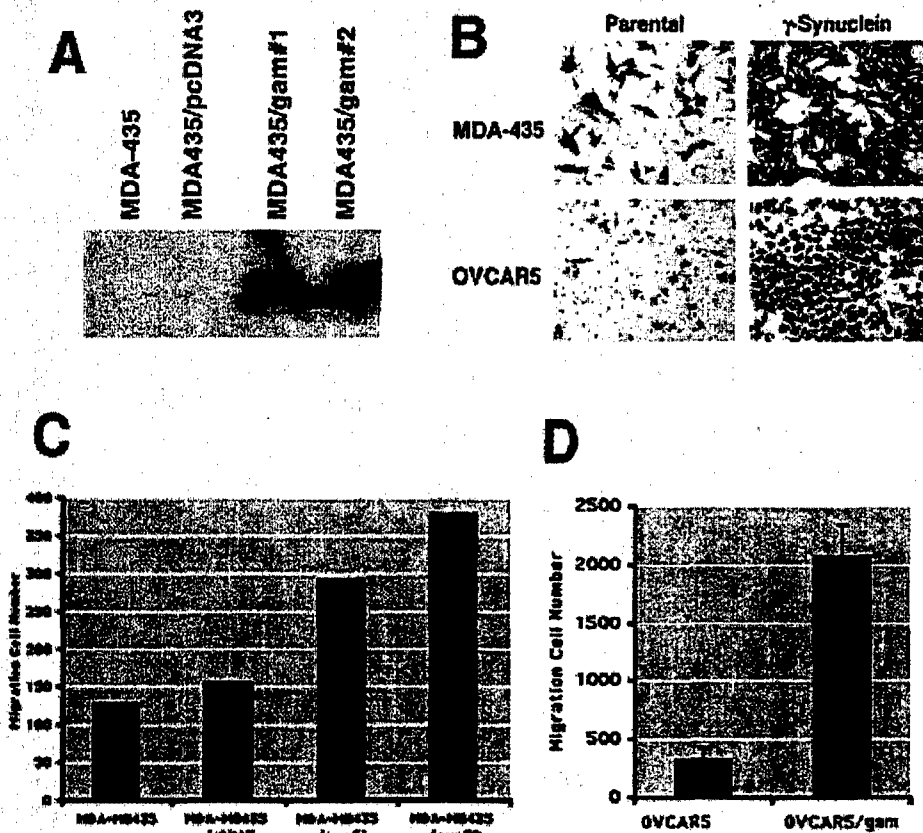
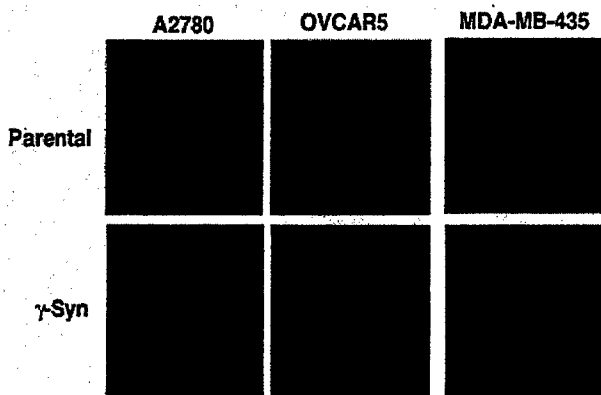


Fig.4. γ -Synuclein enhance cell migration. **A**, γ -synuclein expression in parental MDA-MB-435 cells, and those stably transfected with pcDNA3 vector alone, or with CMV- γ -synuclein (two clones were shown here). **B** through **D**, Boyden chamber assay for cell migration. Cells migrated to the lower chamber were stained (**B**) and counted (**C**, **D**).



members play a pivotal role in regulating stress fiber and focal adhesion formation. In cells over-expressing γ -synuclein, we found at least one major Rho/Rac/Cdc42 member is activated although the protein levels of these proteins were not affected (Fig. 6).

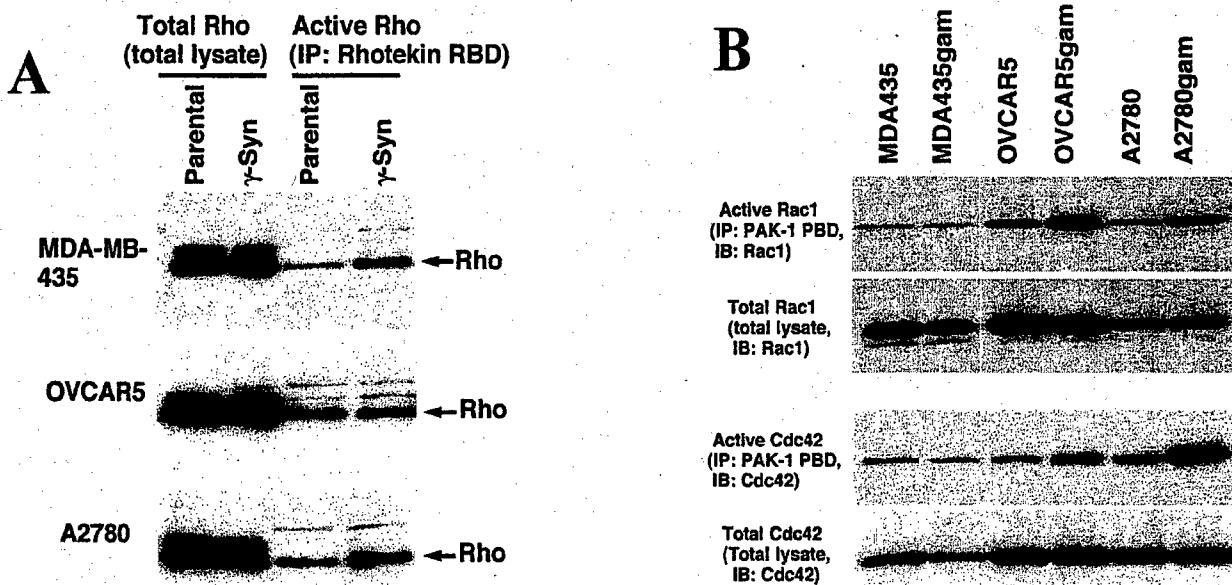


Fig. 6. Enhanced activation of Rho (A) and Rac/Cdc42 (B) in cancer cells over-expressing γ -synuclein. A, whole cell lysate (2 mg protein) were immunoprecipitated with Rhotekin Rho binding domain and analyzed by Western blot with anti-Rho antibody. B, whole cell lysate (2 mg protein) were immunoprecipitated with PAK1 PBD and analyzed by Western blot with anti-Rac and anti-Cdc42 antibodies, respectively.

Requirements of Rho/Rac/Cdc42 and ERK for γ -synuclein-enhanced cell motility-

As described above, over-expression of γ -synuclein also leads to enhanced activation of ERK. To determine whether and to what extent Rho/Rac/Cdc42 or ERK kinase contribute to cell motility and invasiveness, parental cancer cells and their corresponding γ -synuclein over-expressing cells were treated with specific inhibitors. When treated with *C. difficile* toxin B which can inactivate most, if not all, members of the Rho family, and U0126, an inhibitor of ERK activation, the cell migration in both parental and γ -synuclein over-expressing cells were almost completely blocked (Fig. 7). These data

indicate that both Rho and ERK pathways are involved in the parental basal level cell migration and the enhanced cell migration induced by γ -synuclein.

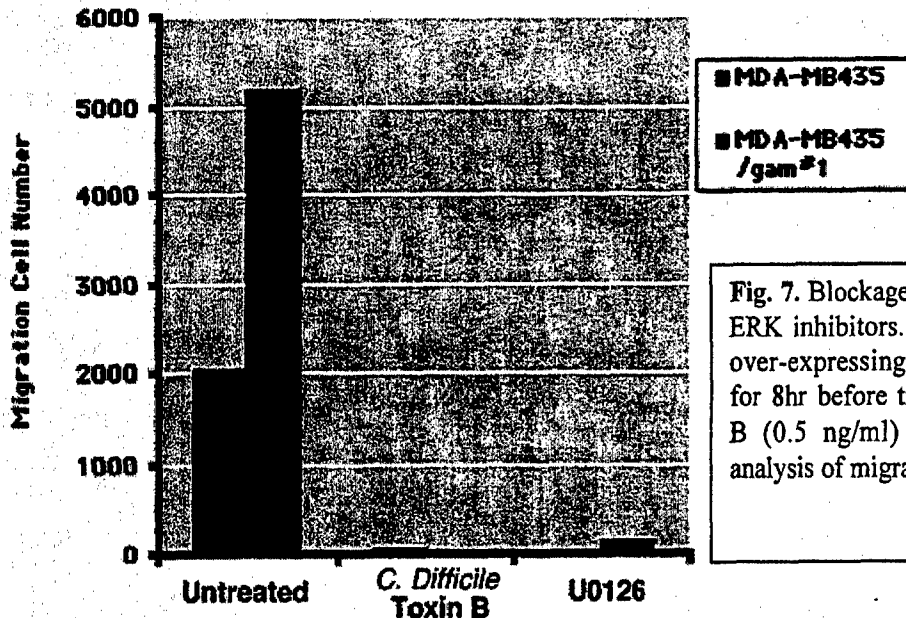


Fig. 7. Blockage of cell migration by Rho and ERK inhibitors. Parental cells or γ -synuclein over-expressing cells were allowed to attach for 8hr before treated with *C. difficile* Toxin B (0.5 ng/ml) or U0126 (10 μ M) before analysis of migrated cells at 52hr.

Down-regulation of JNK activation by γ -synuclein in response to UV - JNK is activated by stress signals including UV which leads to mitochondria mediated apoptosis (Davis, 2000). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ -synuclein was over-expressed or not (Fig. 8). JNK was highly activated in the parental cells

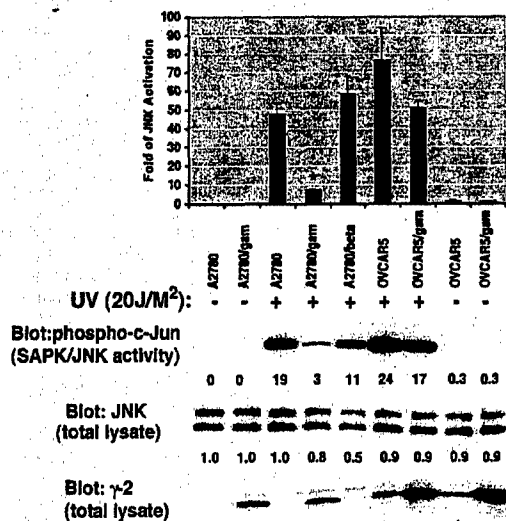


Fig. 8. Inhibition of JNK activation by γ -synuclein in response to UV treatment. A2780, A2780/gam, A2780/beta, OVCAR5, OVCAR5/gam cells were un-treated or treated with UV (20 J/M²) and cells were lysed at 30 min. JNK activities were analyzed by an immunocomplex kinase assay using GST-c-JUN as substrate. The phosphorylated GST-c-JUN by activated JNK was evaluated by immunoblotting with anti-phospho-c-JUN specific antibody. The protein levels of JNK and γ -synuclein were determined by immunoblotting with anti-JNK and γ -2 antibody, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average \pm S.E. of three independent experiments. (*) Represents significant inhibition of JNK activation compared to that in the parental cells ($p < 0.05$).

when treated with UV (Fig. 8). In cells over-expressing γ -synuclein, the activation of JNK was almost completely blocked in A2780/gam cells ($p < 0.05$) and was down-regulated by approximately 50% in OVCAR5/gam cells when treated with UV (Fig. 8) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ -synuclein specific since over-expression of α - and β -synucleins appeared to have little or no effect on the activation of JNK by UV (Fig. 8, and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific, or it could be the high endogenous γ -synuclein

expression in OVCAR5 cells. Collectively, these data indicate that stress-induced activation of JNK can be blocked by γ -synuclein over-expression in a variety of cell lines.

γ -Synuclein may protect paclitaxel (Taxol) induced cell death by regulating JNK and ERK activities.

Based on the data presented above, we hypothesized that γ -synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated γ -synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, cell survival and cell death are regulated by the counter-balance between the survival factors and the apoptotic signaling pathways. Since UV treatment also activates the cell survival pathways ERK [(Rosette and Karin, 1996), and data not shown] and PI3K-AKT (Krasilnikov et al., 1999; Nomura et al., 2001), the initiation of the mitochondria associated caspase pathway may be blocked by the activation of ERK or AKT in A2780 cells. In support of these findings we did not observe the cleavage of the caspase-3 substrate PARP in A2780 cells when treated with UV (data not shown).

We next evaluated the survival of γ -synuclein over-expressing cells in response to Taxol, a commonly used chemotherapeutic drug. In addition to its role in affecting microtubule assembly, Taxol is known to lead to apoptosis via the mitochondria by activating the JNK signaling pathway and Taxol-induced apoptosis can be enhanced by MEK inhibition (Lee et al., 1998; Mandlekar et al., 2000; Wang et al., 1999; Wang et al., 2000). In A2780 cells, Taxol did not affect the basal level activity of ERK or the activation of ERK by γ -synuclein (Fig. 9A). To test the effect of γ -synuclein on cell survival, cells were treated with Taxol for varying lengths of time. At 48hr after treatment, 45-60% of A2780 cells had died, while only 7-15% of A2780/gam cells were dead indicating that Taxol

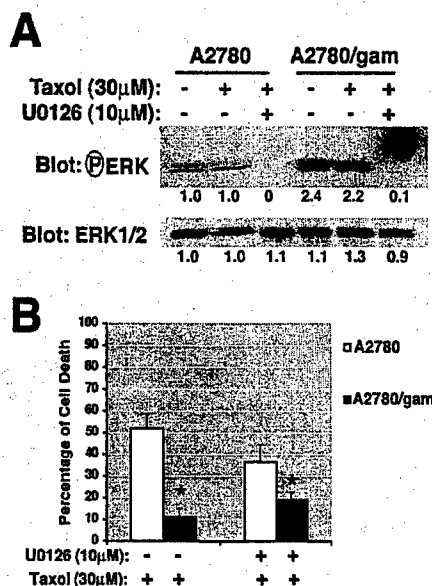
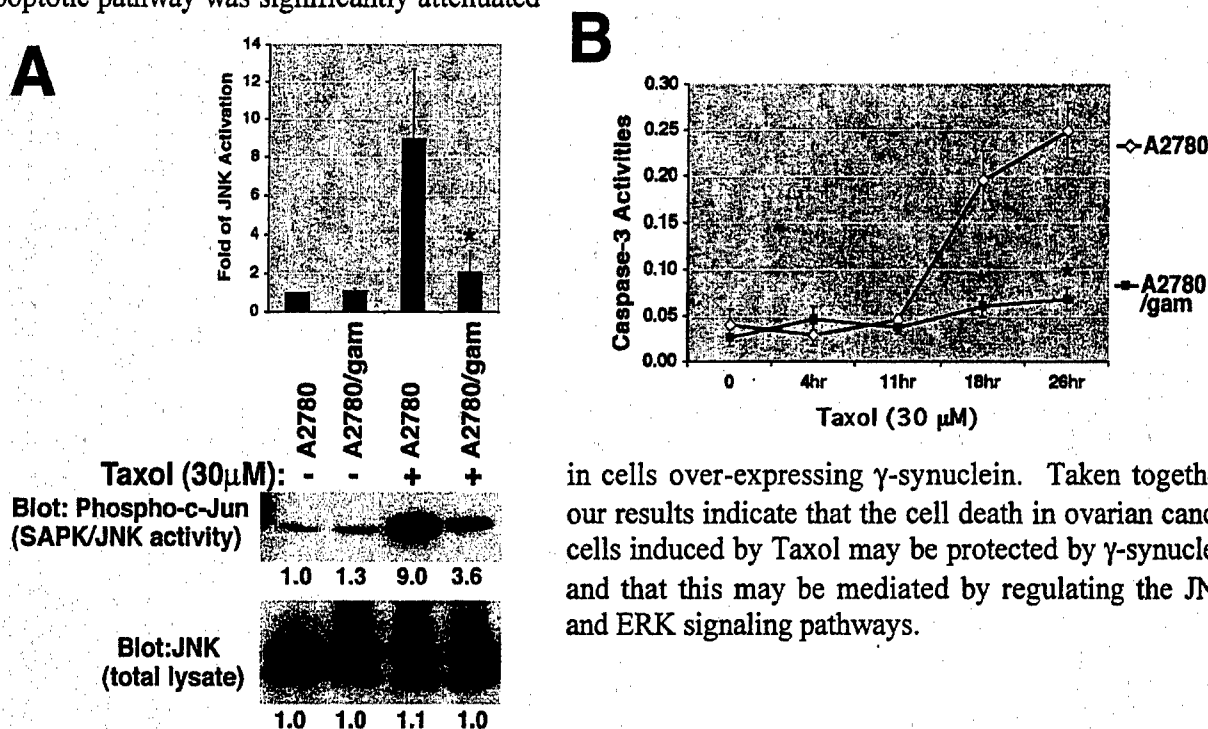


Fig. 9. γ -Synuclein protects cells from paclitaxel (Taxol) induced cell death and is partially mediated by ERK activation. **A**, Taxol does not affect ERK activity or γ -synuclein mediated ERK activation. A2780 or A2780/gam cells pre-treated with or without U0126 (10 μ M) were treated with Taxol (30 μ M) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **B**, cell death induced by Taxol was significantly reduced in cells over-expressing γ -synuclein. A2780 and A2780/gam cells treated with Taxol for 48hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average \pm S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$).

induced cell death can be suppressed by γ -synuclein over-expression (Fig. 9B). When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced by ~25% in A2780 cells but was nearly doubled in A2780/gam cells (Fig. 9B). These data suggest that enhanced cell survival in γ -synuclein over-expressing cells is partially mediated by activation of ERK.

To determine whether the protective role of γ -synuclein on cell survival is also mediated through down-regulating JNK associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (Lee et al., 1998; Wang et al., 1999), JNK was activated in A2780 cells when treated with 30 μ M Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol ($p < 0.05$) (Fig. 10A). In the parental A2780 cells, caspase-3 was highly activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in γ -synuclein over-expressing ovarian cancer cells ($p < 0.05$) (Fig. 10B). These data indicate that Taxol activated JNK mediated caspase apoptotic pathway was significantly attenuated



in cells over-expressing γ -synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by γ -synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

Fig. 10. Taxol activated JNK and caspase-3 apoptotic pathway is blocked by γ -synuclein. **A**, down-regulation of JNK activation by Taxol in cells over-expressing γ -synuclein. Cell lysates from A2780 and A2780/gam cells treated with or without Taxol (30 μ M) for 60 min were assayed for JNK activity (see the legend for Fig. 8 for experimental details). The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average \pm S.E. of three independent experiments. **B**, down-regulation of caspase-3 activity activated by Taxol in A2780/gam cells. A2780 and A2780/gam cells treated with Taxol (30 μ M) for different time lengths were lysed, and approximately 20 μ g protein were incubated with the caspase-3 substrate Ac-DEVD-pNA for 4hr at 37°C. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate reader. The graph represents the average \pm S.E. of three independent experiments. In panels A and B, (*) represents significant difference compared to that in the parental cells ($p < 0.05$).

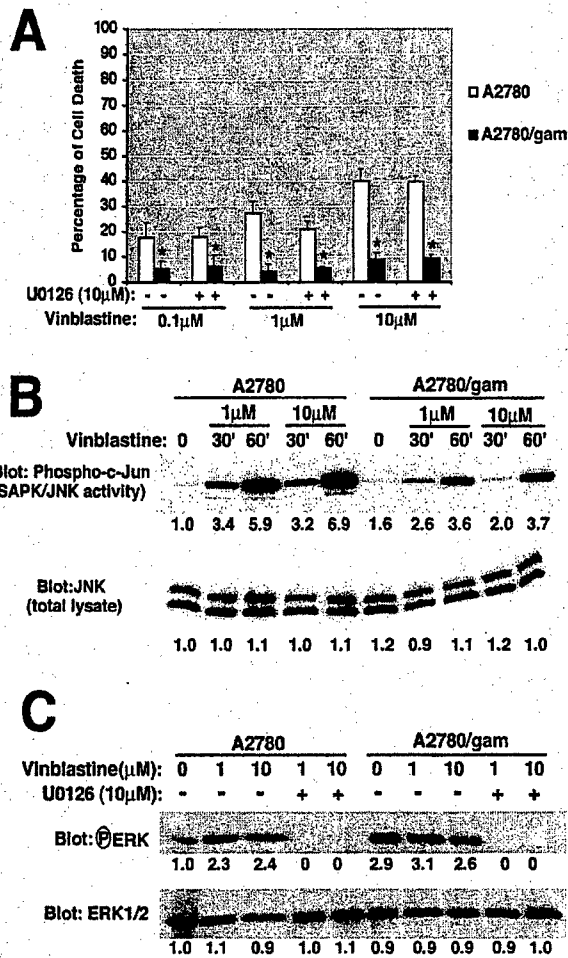
γ -Synuclein over-expression leads to protection from vinblastine but not etoposide induced cell death.

Fig. 11. Vinblastine induced cell death and activation of the MAPK pathways in γ -synuclein over-expressing cells. **A**, the cell death induced by vinblastine was significantly reduced in cells over-expressing γ -synuclein. A2780 and A2780/gam cells treated with vinblastine (0.1, 1, or 10 μ M) for 30 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average \pm S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$). **B**, inhibition of JNK activation by γ -synuclein in response to vinblastine treatment. A2780 and A2780/gam cells were untreated or treated with vinblastine (1 μ M, and 10 μ M) for 30 min and 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in the legend for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C**, the effect of vinblastine on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with vinblastine (1 μ M and 10 μ M) in the absence or presence of U0126 (10 μ M) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

To demonstrate whether the effects of γ -synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents; Taxol binds to microtubule polymers while vinblastine binds to monomers and dimers. When treated with vinblastine (either 0.1, 1.0, or 10 μ M for 30 hr), cell death in A2780/gam cells was significantly lower ($p < 0.05$ for all the three concentrations tested) as compared to the parental cells (Fig. 11A). Consistent with other studies using a variety of tumor cell lines (Fan et al., 2000; Osborn and Chambers, 1996; Stone and Chambers, 2000; Wang et al., 1999), vinblastine strongly activate JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by γ -synuclein over-expression (Fig. 11B). Furthermore, we observe that treatment with vinblastine results in a two-fold increase in phosphorylated ERK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (Fig. 11B). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (Fig. 11A & C). These data

indicate that suppression of vinblastine-induced cell death by γ -synuclein may be mediated by inhibition of JNK activation.

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (Anderson et al., 1999; Gibson et al., 1999; Jarvis et al., 1999; Osborn and Chambers, 1996). When treated with 1, 10, or 100 μ M of etoposide for 56 hrs, there was no significant difference in cell survival between A2780 and A2780/gam cells (Fig. 12A). As might be predicted, JNK was not activated in response to etoposide treatment (Fig. 12B). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down-regulated within 30 min of treatment with 10 or 100 μ M of etoposide (Fig. 12C). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and γ -synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.

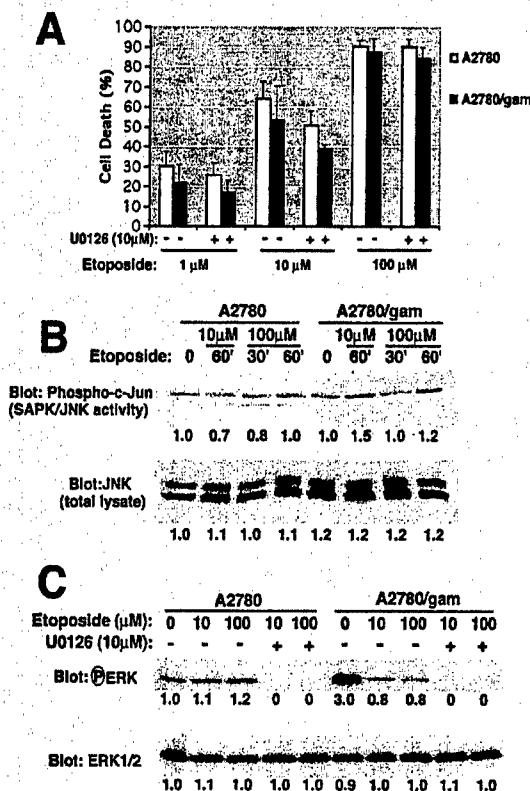


Fig. 12. Effects of γ -synuclein over-expression on etoposide induced cell death and activation of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express γ -synuclein. A2780 and A2780/gam cells treated with etoposide (1 μ M, 10 μ M, 100 μ M) for 56 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average \pm S.E. of three independent experiments. B, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 μ M, and 100 μ M) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. C, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 μ M and 100 μ M) in the absence or presence of U0126 (10 μ M) for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results.

Summary. Based on the data we presented above, we proposed the following model (Fig. 13) that γ -synuclein may enhance cell migration and metastasis, promote cell survival and inhibit apoptosis in tumor progression by modulating Rho and MAPK pathways.

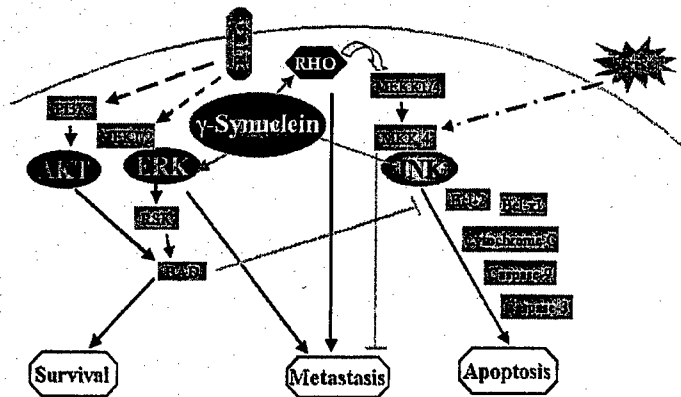


Figure 13. Diagram illustrating the effects of γ -synuclein on relevant signaling transduction pathways and their effects on metastasis, cell survival and apoptosis.

C- KEY RESEARCH ACCOMPLISHMENTS:

C.1. "Gamma synuclein promotes a metastatic phenotype in breast and ovarian tumor cells by modulating the Rho signal transduction pathway".

1.a. Overexpression of γ -synuclein leads to constitutive activation of ERK and Rho/Rac/Cdc42 and down-regulation of JNK activation in response to stress signals or chemotherapy drugs.

1.b. Overexpression of γ -synuclein induces stress fiber formation and enhances cell migration. Both the basal level and the enhanced cell migration require the activities of both the ERK and Rho/Rac/Cdc42 kinases.

1.c. Overexpression of γ -synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway.

D-REPORTABLE OUTCOMES (1/2002 to present):

D.1.. "Gamma synuclein promotes a metastatic phenotype in breast and ovarian tumor cells by modulating the Rho signal transduction pathway".

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1.a. Abstracts

*Pan, Z-Z., Bruening, W., Giasson, B.I., Lee, V.M., **Godwin, A.K.** γ -Synuclein is over-expressed in breast and ovarian cancers and promotes tumor cell survival by inhibiting stress-induced apoptosis. Proceedings of American Association of Cancer Research, 43:26, 2002.

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Capo-chichi, C.D., Rula, M.E., Smedberg, J.L., Yang, D-H., **Godwin, A.K.**, Xu, X-X. GATA4 and GATA-6 mediate retinoic acid-induced disabled-2 expression in mouse embryonic carcinoma cells. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002.

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Prowse, A.H., Slater, C.M., **Godwin, A.K.** Analysis of hnRNP A1 and RBM8/Y14 expression in tumors; implications for aberrant RNA processing during tumorigenesis. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002.

*Pan, Z-Z., Bruening, W., Giasson, B.I., Lee, V.M., **Godwin, A.K.** γ -Synuclein may render cancer cells resistant to paclitaxel and nitric oxide induced apoptosis by modulating MAPK pathways. The Seventh Annual Postdoctoral Research Conference, Philadelphia, 2002.

1.b. Publications

Okamoto, I., Tsuiki, H., Kenyon, L., **Godwin, A.K.**, Emlet, D.R., Holgado-Madruga, M., Guha, A., Ushio, U., Saya, H., Wong, A.J. Proteolytic Cleavage of CD44 Adhesion Molecule in Multiple Human Tumors. *American Journal of Pathology*, 160:441-447, 2002.

Roberts, D., Williams, S.J., Cvetkovic, D., Weinstein, J.K., **Godwin, A.K.**, Johnson, S.W., Hamilton, T.C. Decreased Expression of Retinol-Binding Proteins is Associated with malignant transformation of the ovarian surface epithelium. *DNA and Cell Biology*, 21:11-19, 2002.

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Capo-chichi, C.D., Smith, E.R., Yang, D-H., Roland, I.H., Vanderveer, L., Cohen, C., Hamilton, T.C., **Godwin, A.K.**, and Xu, X-X. Dynamic alterations of extracellular environment of ovarian surface epithelial cells in pre-malignant transformation, tumorigenicity, and metastasis. *Cancer*, in press, 2002.

*Pan, Z-Z., Bruening, W., Giasson, B., Lee, V., **Godwin, A.K.** γ -Synuclein promotes cancer cell survival and inhibits stress-induced apoptosis by modulating MAPK pathways. *JBC*, in press (on line as of July 16, 2002).

E-CONCLUSIONS:

E.1. "Gamma synuclein promotes a metastatic phenotype in breast and ovarian tumor cells by modulating the Rho signal transduction pathway".

In these studies, we found that γ -synuclein can interact with two major MAPKs, i.e., ERK and JNK1. Over-expression of γ -synuclein may lead to enhanced activity of ERK and down-regulation of JNK activation in response to stress and chemotherapy drugs. Rho/Rac/Cdc42 pathway is also activated in cells over-expressing γ -synuclein. Activation of both the Rho/Rac/Cdc42 and ERK pathways are required for the enhanced cell migration in γ -synuclein over-expressing cells. Over-expression of γ -synuclein may render cancer cells resistant to Taxol and vinblastine by modulating ERK cell survival pathway and JNK-mitochondria-Caspase 9/3 apoptotic pathway. Taken together, these data indicate that γ -synuclein may promote tumorigenesis by enhancing cell motility through modulating Rho/Rac/Cdc42 and ERK pathways, and promoting cell survival and inhibiting apoptosis through modulating ERK cell survival and JNK-mitochondria-caspase9/3 apoptotic pathways. Since γ -synuclein is aberrantly expressed in the majority of late-stage breast and ovarian cancers but is not expressed in normal breast and ovarian epithelial cells, γ -synuclein may represent a very promising therapy target for these diseases.

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APPENDICES:

Pan, Z-Z, Bruening, W., Giasson, B., Lee, V., **Godwin, A.K.** γ -Synuclein promotes cancer cell survival and inhibits stress-induced apoptosis by modulating MAPK pathways. JBC, in press.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

γ -Synuclein Promotes Cancer Cell Survival and Inhibits Stress- and Chemotherapy Drug-Induced Apoptosis by Modulating MAPK Pathways*

Zhong-Zong Pan[‡], Wendy Bruening^{‡,§}, Benoit I. Giasson[¶], Virginia M-Y. Lee[¶], and Andrew K. Godwin^{‡}**

[‡] Department of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111

[§] Present address: Health Technology Assessment Information Service, Plymouth Meeting, PA 19462

[¶] Center for Neurodegenerative Diseases, University of Pennsylvania School of Medicine, 3600 Spruce Street, Philadelphia, PA 19104

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** To whom correspondence should be addressed: Dept. of Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Rm. W322, Philadelphia, PA 19111. Tel.: 215-728-2205; Fax: 215-728-2741; E-mail: A_Godwin@fccc.edu.

¹ The abbreviations used are: MAPK, mitogen-activated kinase; BCSG1, breast cancer-specific gene 1; ERK, extracellular signal-regulated protein kinase; JNK, c-JUN N-terminal kinase; MEK, MAP kinase kinase; NACP, the precursor of NAC (non-A component of AD amyloid); LBs, Lewy bodies; AD, Alzheimer's disease; DLB, dementia with LBs.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

SUMMARY

Synucleins are a family of highly conserved small proteins predominantly expressed in neurons. Of the synucleins, α -synuclein is the best characterized because of its potential significance in neurodegenerative diseases. Recently we and others have found that γ -synuclein is dramatically up-regulated in the vast majority of late-stage breast and ovarian cancers and that γ -synuclein over-expression can enhance tumorigenicity. In the current study, we have found that γ -synuclein is associated with two major mitogen-activated kinases (MAPK), i.e., extracellular signal-regulated protein kinases (ERK1/2) and c-Jun N-terminal kinase 1 (JNK1) and have shown that over-expression of γ -synuclein leads to constitutive activation of ERK1/2 and down-regulation of JNK1 in response to a host of environmental stress signals including UV, arsenate, and heat shock. We also tested the effects of γ -synuclein on apoptosis and activation of JNK and ERK in response to several chemotherapy drugs. We have found that γ -synuclein expressing cells are significantly more resistant to the chemotherapeutic drugs paclitaxel and vinblastine as compared to the parental cells. The resistance to paclitaxel can be partially obliterated when ERK activity is inhibited using a MEK1/2 inhibitor. Activation of JNK and its downstream caspase-3 by paclitaxel or vinblastine is significantly down-regulated in γ -synuclein expressing cells, indicating that paclitaxel or vinblastine activated apoptosis pathway is blocked by γ -synuclein. In contrast to paclitaxel and vinblastine, etoposide does not activate JNK and γ -synuclein over-expression has no apparent effect on this drug-induced apoptosis. Taken together, our data indicate that oncogenic activation of γ -synuclein contributes to the development of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain chemotherapeutic drugs.

INTRODUCTION

Breast carcinoma is the second leading cause of cancer related deaths in women of the western world. In the United States alone over 180,000 new cases are diagnosed annually and more than 40,000 women die from this disease each year (1). Epithelial ovarian cancer continues to be the leading cause of death from gynecologic malignancies in the United States (1,2). One woman in 70 in the U.S. will develop ovarian cancer in her lifetime, and one woman in 100 will die of this disease. Breast and ovarian cancer etiology are multifactorial; involving environmental factors, hormones, genetic susceptibility, and genetic changes during progression. Both cancers are a heterogeneous group of tumors with no unifying molecular alteration yet identified. A certain number of breast and ovarian cancer cases (~5 to 10%) are attributed to inherited mutations in highly penetrant breast cancer susceptibility genes, such as *BRCA1* and *BRCA2* [reviewed in (3)]. However, the majority of the tumors occur in women with little or no family history and the molecular basis of these sporadic cancers is still poorly defined.

In an effort to identify other genes involved in the development and/or progression of breast and ovarian cancer, we and others used differential gene expression approaches and have found that γ -synuclein, initially termed breast cancer-specific gene 1 (BCSG1), is up-regulated in the majority of late-stage breast (4,5) and ovarian cancer [Bruening, 2000 #16; Pan and Godwin, unpublished data]. In addition, we showed that there was a correlation between γ -synuclein expression in breast ductal carcinomas and the staging of the cancer suggesting that γ -synuclein may be a potential marker for both late stage breast and ovarian cancer. Additional studies have revealed that γ -synuclein over-expression leads to increased invasiveness of breast tumor cells (6) and stimulated cell proliferation (7).

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

Synucleins are a family of small, highly soluble proteins that are predominantly expressed in neurons. The functions of the synucleins are not entirely understood. There are four known members: α -synuclein [also referred to as synelfin or non- $A\beta$ component of Alzheimer's disease (AD) amyloid precursor protein (NACP)], and β -synuclein [also referred to as phosphoneuroprotein 14 (PNP-14)] are neuronal proteins primarily expressed in brain and are predominantly found at axonal terminals. γ -Synuclein [also known as persyn] is predominantly expressed in certain regions of the peripheral nervous system, such as dorsal root ganglia and trigeminal ganglia. Synoretin, the newest member of the synuclein family is expressed at high levels in the retina and at lower levels in the brain (8,9). The synuclein proteins contain several repeated domains that display variations of a KTKEGV consensus sequence. The β -synuclein protein contains five of these domains, whereas the α - and γ -synucleins have six. Interestingly, the third domain of each protein is completely conserved and this same type of domain is present in proteins of the Rho family (10). Another type of organization of the synuclein proteins that has been noted is an 11-residue repeat. This motif, repeated six to seven times in the amino-terminal portion of the protein, is reminiscent of the amphipathic α -helical domains of the apolipoproteins and suggests possible lipid binding properties (11).

The γ -synuclein gene maps to 10q23, is composed of five coding exons, and is transcribed into an ~1 kb mRNA (8). The human γ -synuclein is 127 amino acids long, and is 87.7% and 83.3% identical to the mouse and rat proteins, respectively. In addition, comparison of the amino acid sequences indicates that γ -synuclein is highly homologous to α -synuclein and β -synuclein except for the last 27 amino acids of γ -synuclein. Overall, γ -synuclein shares 54%, 56%, and 84% amino acid sequence identity with α -synuclein, β -synuclein, and synoretin, respectively

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

(8,9).

Among the synucleins, α -synuclein is the best characterized because of its significant role implicated in neurodegenerative diseases (12). Mutations in the α -synuclein gene have been identified in rare kindreds with Parkinson's disease (12-14) and intracytoplasmic aggregates comprised of α -synuclein fibrils are characteristic of several neurodegenerative diseases as exemplified by the intraneuronal Lewy bodies (LBs), neuroaxonal spheroids and dystrophic neurites (i.e. Lewy neurites) that are prominent in PD, LB variant of Alzheimer's disease (AD), and dementia with LBs (DLB) (14-19).

The normal physiological functions of synucleins are not well characterized. The N-terminal portion of α -synuclein (residues 1-61) shares 40% amino acid homology with members of the 14-3-3 protein family (20). The 14-3-3 family of proteins helps regulate many different signal transduction pathways, and is thought to act by directly binding to various protein kinases and bringing them into close proximity with substrate and regulatory proteins. 14-3-3 proteins bind to phospho-Ser residues critical for the functions of many kinases and phosphatases that are involved in diverse cell functions (21-24). Like the synucleins, 14-3-3 proteins are ubiquitously expressed in the brain and have been shown to associate in a chaperone-like manner with PKC, BAD, ERK, and RAF-1 (16,21). α -Synuclein binds 14-3-3 as well as to PKC, BAD, ERK and the microtubule associated protein tau (25). In addition to shared regions of homology to 14-3-3, α -synuclein, as well as β - and γ -synuclein also appears to act as a protein chaperone, at least *in vitro*, by disrupting protein aggregation (26).

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

To help further unravel the function of γ -synuclein and establish its role in the oncogenesis of breast and ovarian cancer, we searched for proteins that could interact with γ -synuclein and identified the MAP kinases ERK1/2 and JNK1. In this study we provide evidence that γ -synuclein contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways. In addition, we observed that paclitaxel- or vinblastine-induced cell death is protected by γ -synuclein indicating that chemotherapeutic drugs that take effect through activating the JNK apoptosis pathway may not be effective for cancer with high γ -synuclein expression.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

EXPERIMENTAL PROCEDURES

Reagents and Antibodies – Paclitaxel, vinblastine, and etoposide were purchased from Sigma (St. Louis, MO). The MEK1/2 inhibitor U0126 was purchased from Promega (Madison, WI). Anti-ERK1, anti-ERK2, anti-JNK1 antibodies and normal IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-ERK1/2 and anti-PARP antibodies were obtained from Cell Signaling (Beverly, MA). The mouse antibody Syn303 was raised to recombinant human α -synuclein, but recognizes α -, β -, and γ -synuclein. γ -2 is a rabbit polyclonal antibody raised to recombinant human γ -synuclein that specifically recognizes human γ -synuclein (27). Mouse monoclonal antibodies Syn204 and Syn207 were raised against human α - and β -synucleins, respectively.

Cell culture and transfection - Ovarian cancer cell lines A2780 and OVCAR5 were maintained in 10% FBS DMEM and 10% FBS RPMI 1640, respectively. HEK 293, human embryonic kidney cells, were maintained in 10% FBS DMEM supplemented with Na-pyruvate and non-essential amino acids. To create the CMV plasmid for establishing stable cell lines over-expressing human α -, β - or γ -synuclein, human cDNAs were amplified by PCR and subcloned into pcDNA3 (Invitrogen). GenePorter Transfection Reagent (GTS Inc, San Diego, CA) was used for transfection and stable cell lines were selected by G418 (Gibco BRL). Expression of human α -, β - or γ -synuclein was confirmed by immuno-blotting with Syn204, Syn207, or γ -2 antibody, respectively.

Cell treatment with UV and other stress signals or chemotherapeutic drugs - For UV treatment, cells at 70-80% confluence were washed once with PBS before UV irradiation (254

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

nm, 20 J/m²). Complete medium was added to the plates after treatment. The intensity of the UV light source was measured with a BLAK-RAY meter (UVP, Inc., San Gabriel, CA) prior to each experiment. Heat-shock was carried at 42°C for 15 min. 50 μ M Na-arsenite was used to treat the cells for 6 hr. Paclitaxel, vinblastine, etoposide, and U0126 were dissolved in DMSO and cells were treated with various concentrations of the drugs as indicated in each experiment.

Co-Immunoprecipitation - Cells at 70-80% confluence were washed twice with ice-cold D-PBS before scraping on ice with Lysis Buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2.5 mM Na-pyrophosphate; 1 mM Na- β -glycerophosphate; 5 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 1% Triton X-100, and 1 tablet of protease inhibitor cocktail (Roche, Indianapolis, IN) per 40 ml lysis buffer]. Cellular debris was removed by centrifugation (14,000 x g for 15 min at 4°C) and precleared with protein G-agarose (Gibco BRL, Rockville, MD). Protein concentrations were determined with Bio-Rad DC protein assay reagents. Syn303 (3 μ l ascites) or control IgG (3 μ g) were pre-incubated in 500 μ l of PBS with 50 μ l of protein-G agarose overnight at 4°C, and washed twice with PBS before incubation with 300 μ g of total cellular lysate for 4 hr at 4°C. The beads were washed 4 x with the lysis buffer, resuspended in 50 μ l 2 x SDS sample buffer before boiling for 5 min. 10 μ l immunoprecipitates were separated by SDS-PAGE electrophoresis on 4-20% linear gradient Tris-HCl ready gels (Bio-Rad).

Immuno-blotting and data quantification - Proteins separated on SDS-PAGE gels were transferred onto Immobilon-P PVDF membrane (Millipore, Bedford, MA). The primary antibodies were diluted 1:1000, and the HRP-conjugated second antibodies were diluted

γ-Synuclein promotes cancer cell survival by affecting MAPK pathways

1:10,000 (Amersham, Piscataway, NJ). NEN Renaissance Enhanced Luminol Reagents (Boston, MA) were used as substrates for detection. For re-use of the same membrane with another primary antibody, Restore Western Blot Stripping buffer (Pierce, Rockford, IL) was used to strip the membrane. The results of immuno-blotting were quantitated using the NIH Image for the integrated density of each band.

JNK-kinase activity assay - The kinase activity of JNK was measured using the SAPK/JNK assay kit (Cell Signaling Technologies). Briefly, 250 μ l cell lysate (1 μ g/ μ l protein) was incubated with 2 μ g c-JUN fusion protein beads (in 20 μ l) overnight at 4°C. After washing, the proteins on the beads were incubated in the kinase reaction buffer supplemented with 100 μ M ATP for 30 min at 30°C. To measure the JNK activity, the phosphorylated c-JUN was detected by SDS-PAGE and immunoblotting with the specific antibody (Cell Signaling Technologies).

Cell viability assay - Cell viability was determined by Trypan blue exclusion assay and/or WST-1 assay. For Trypan blue assay, cells were stained with 0.2% Trypan blue for 2-5 min. The number of viable cells (non-stained) and dead cells (stained) were counted under microscope using a cell hemocytometer. For WST-1 assay, cells under different culture conditions were incubated with WST-1 (Roche) for 4 hr. Cleavage of WST-1 to formazan was monitored at 450 nm using a microplate reader.

Caspase activity assay - Colorimetric CaspACE assay (Promega) was used to detect the caspase-3 activity. Briefly, pNA released from the substrate Ac-DEVD-pNA by caspase-3 in the cell lysate was monitored at 405 nm using a microplate reader.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

Statistical analysis – Where indicated, a two-tailed student t-test was used to test for significance.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

RESULTS

Expression of synuclein in tumor cell lines - We have previously reported that γ -synuclein is highly expressed in the vast majority of late-stage breast and ovarian tumors (4), suggesting a potentially important role for γ -synuclein in the development of these diseases. To help unravel the function of γ -synuclein, we established several *in vitro* models. The ovarian tumor cell lines A2780 and OVCAR5, which express low levels of γ -synuclein as well as kidney HEK 293 cells which do not express detectable levels of γ -synuclein were transfected with CMV- γ -synuclein or with vector alone and were selected with G418. Resistant colonies were screened by Western blotting for stable expression of γ -synuclein protein and positive colonies were pooled into A2780gam, OVCAR5gam, and 293gam cell lines (Fig. 1). Cell lines stably expressing α -synuclein and β -synuclein were also derived from A2780 cells as described for the γ -synuclein expressing lines (Fig. 1). Like A2780gam, OVCAR5gam, 293gam cells, there were no obvious alterations in cell doubling time of the A2780alpha or A2780beta cell lines (data not shown).

γ -Synuclein interacts with ERK and JNK MAP kinases in cancer cells - γ -Synuclein has recently been reported to bind directly to the ERK2 kinase (28). Therefore, we evaluated whether γ -synuclein could also interact with the ERK kinases as well as other MAPKs. By co-immunoprecipitation approaches, we were able to demonstrate a novel association of γ -synucleins with ERK1/2 and JNK1 kinase, but not with the p38 kinase (Fig. 2). We also confirmed that α -synuclein is associated with ERK1/2 as well with JNK1 (Fig. 2), which is consistent with the recently studies using neuro2a, a neuronal cell line (29). These data indicate that γ - and α -synuclein can interact with ERK1/2 and JNK1 in cancer cells that over-express these proteins.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

Elevated activity of ERK in cells over-expressing γ -synuclein - We next evaluated whether these protein interactions would affect the activity of ERK1/2 and/or JNK1 (see below). In A2780 and OVCAR5 cancer cells over-expressing γ -synuclein, the activated ERK1/2 was increased 2-3 fold as evidenced by immunoblotting with an anti-phospho-ERK specific antibody (Fig. 3). In contrast, α - and β -synucleins appeared to have little or no effect on the activity of ERK1/2 (Fig. 3A) although α -synuclein was also found to be associated with ERK (as described above and shown in Fig. 2) in A2780 cells. In HEK 293 cells, the basal level of ERK activation is undetectable and γ -synuclein over-expression does not increase its activation level (Fig. 3B). Structural analysis indicate that γ -synuclein does not contain any kinase domain, suggesting that the activation of ERK is mediated by other kinase. Since MEK1/2 is required for the activation of ERK1/2 in response to many mitogens, we determined whether MEK1/2 is still required for γ -synuclein mediated activation of ERK1/2. When cells over-expressing γ -synuclein were treated with the MEK1/2 inhibitor U0126, the activation of ERK1/2 was suppressed (Fig. 4A). We further studied the relation of γ -synuclein-ERK interaction and the activation status of ERK1/2. In cells treated with U0126 or serum-starved, the association of γ -synuclein and ERK1/2 was still present (Fig. 4B). These data indicate that γ -synuclein may be constitutively associated with ERK1/2, which could facilitate the activation of ERK by MEK1/2 and lead to the constitutive activation of ERK1/2 in cells over-expressing γ -synuclein.

Down-regulation of JNK activation by γ -synuclein in response to different stress signals -

JNK is activated by stress signals including UV which leads to mitochondria mediated apoptosis (30). The basal level of JNK activity in A2780 and OVCAR5 ovarian cancer cells is very low in untreated cells whether γ -synuclein was over-expressed or not (Fig. 5). JNK was highly

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

activated in the parental cells when treated with UV (Fig. 5). In cells over-expressing γ -synuclein, the activation of JNK was almost completely blocked in A2780/gam cells ($p < 0.05$) and was down-regulated by approximately 50% in OVCAR5/gam cells when treated with UV (Fig. 5) or heat-shock (data not shown). The blockage of JNK activation by UV appears to be γ -synuclein specific since over-expression of α - and β -synucleins appeared to have little or no effect on the activation of JNK by UV (Fig. 5, and data not shown). The inhibition of JNK activation in OVCAR5/gam cells is much less than that in A2780/gam cells. The cause of this difference could either be cell-type specific, or it could be the high endogenous γ -synuclein expression in OVCAR5 cells. Similarly, the activation of JNK by sodium arsenate was blocked to different extents by γ -synuclein in 293/gam, OVCAR5/gam, and A2780/gam cells (Fig. 6). Collectively, these data indicate that stress-induced activation of JNK can be blocked by γ -synuclein over-expression in a variety of cell lines.

γ -Synuclein may protect paclitaxel (Taxol) induced cell death by regulating JNK and ERK activities - Based on the data presented above, we hypothesized that γ -synuclein may contribute to cancer cell survival by up-regulating the ERK cell survival pathway and by suppressing the JNK apoptosis pathway under adverse conditions. However, when we treated γ -synuclein over-expressing cells with UV, significant differences in cell survival between A2780 and A2780/gam cells were not observed (data not shown). The reason for this lack of difference is not readily apparent. However, cell survival and cell death are regulated by the counter-balance between the survival factors and the apoptotic signaling pathways. Since UV treatment also activates the cell survival pathways ERK [(31), and data not shown] and PI3K-AKT (32,33), the initiation of the mitochondria associated caspase pathway may be blocked by the activation of ERK or AKT

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

in A2780 cells. In support of these findings we did not observe the cleavage of the caspase-3 substrate PARP in A2780 cells when treated with UV (data not shown).

We next evaluated the survival of γ -synuclein over-expressing cells in response to Taxol, a commonly used chemotherapeutic drug. In addition to its role in affecting microtubule assembly, Taxol is known to lead to apoptosis via the mitochondria by activating the JNK signaling pathway and Taxol-induced apoptosis can be enhanced by MEK inhibition (34-36). In A2780 cells, Taxol did not affect the basal level activity of ERK or the activation of ERK by γ -synuclein (Fig. 7A). To test the effect of γ -synuclein on cell survival, cells were treated with Taxol for varying lengths of time. At 48hr after treatment, 45-60% of A2780 cells had died, while only about 7-15% of A2780/gam cells were dead indicating that Taxol induced cell death can be rescued by γ -synuclein over-expression (Fig. 7B). When ERK activation was inhibited using the MEK1/2 inhibitor U0126, the cell death was reduced by ~25% in A2780 cells but was nearly doubled in A2780/gam cells (Fig. 7B). These data suggest that enhanced cell survival in γ -synuclein over-expressing cells is partially mediated by activation of ERK.

To determine whether the protective role of γ -synuclein on cell survival is also mediated through down-regulating JNK associated apoptotic pathway(s), caspase-3 activity was monitored at different time points after Taxol treatment. Consistent with the data in other ovarian and breast cancer cell lines (34,36), JNK was activated in A2780 cells when treated with 30 μ M Taxol. The caspase-3 substrate PARP was found cleaved in cells treated with Taxol (data not shown). In A2780/gam cells, the activity of JNK was significantly inhibited following treatment with Taxol ($p < 0.05$) (Fig. 8A). In the parental A2780 cells, caspase-3 was highly

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

activated following drug treatment. By contrast, the activation of caspase-3 by Taxol treatment was significantly reduced in γ -synuclein over-expressing ovarian cancer cells ($p < 0.05$) (Fig. 8B). These data indicate that Taxol activated JNK mediated caspase apoptotic pathway was significantly attenuated in cells over-expressing γ -synuclein. Taken together, our results indicate that the cell death in ovarian cancer cells induced by Taxol may be protected by γ -synuclein and that this may be mediated by regulating the JNK and ERK signaling pathways.

γ -Synuclein over-expression leads to protection from vinblastine but not etoposide

induced cell death – To demonstrate whether the effects of γ -synuclein on cell survival were specific to Taxol or were a general mechanism of drug resistance, we evaluated two additional chemotherapeutic agents, i.e., vinblastine and etoposide. Both Taxol and vinblastine are microtubule-interfering agents; Taxol binds to microtubule polymers while vinblastine binds to monomers and dimers. When treated with vinblastine (either 0.1, 1.0, or 10 μ M for 30 hr), cell death in A2780/gam cells was significantly lower ($p < 0.05$ for all the three concentrations tested) as compared to the parental cells (Fig. 9A). Consistent with other studies using a variety of tumor cell lines (36-39), vinblastine strongly activate JNK in A2780 cells. However, this activation of JNK by vinblastine was significantly inhibited by γ -synuclein over-expression (Fig. 9B). Furthermore, we observe that treatment with vinblastine results in a two-fold increase in phosphorylated ERK1/2 in A2780 cells. This enhancement in activated ERK levels was not observed in the A2780/gam cells (Fig. 9B). Unlike our results for Taxol, inhibition of ERK phosphorylation by U0126 did not significantly affect the cell death in the parental cells or A2780/gam cells (Fig. 9A & C). These data indicate that suppression of vinblastine-induced cell death by γ -synuclein may be mediated by inhibition of JNK activation.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

We also evaluated etoposide, a DNA damage agent that has also been shown to induce JNK activation in some cell lines (37,40-42). When treated with 1, 10, or 100 μ M of etoposide for 56 hrs, there was no significant difference in cell survival between A2780 and A2780/gam cells (Fig. 10A). As might be predicted, JNK was not activated in response to etoposide treatment (Fig. 10B). Furthermore, etoposide treatment did not result in ERK activation in A2780 cells. However, surprisingly the constitutive phosphorylated ERK levels observed in A2780/gam cells were significantly down-regulated within 30 min of treatment with 10 or 100 μ M of etoposide (Fig. 10C). In the presence of the MEK inhibitor U0126, cell death induced by etoposide was reduced in both the parental and γ -synuclein over-expression cells, but statistical analysis indicated these differences were not statistically significant.

DISCUSSION

Synucleins are highly soluble proteins and their biological and biochemical functions are not entirely understood. Previous studies have suggested that they may be involved in neuron development and function (43). The involvement of γ -synuclein in human neoplastic diseases came to light when γ -synuclein was isolated from a human breast tumor cDNA library and was shown to be over-expressed in infiltrating ductal carcinomas (4,5) and ovarian cancer (4). Additional studies have suggested that γ -synuclein may be involved in enhancing cell motility and metastasis, in breast (6) and ovarian (Bruening and Godwin, unpublished data) cancer cells as analyzed both *in vitro* and in nude mouse models *in vivo*. We have found that oncogenic activation of γ -synuclein is independent of gene mutations or gene amplification. We have recently reported that hypomethylation of the γ -synuclein gene CpG island promotes its aberrant expression in breast and ovarian carcinomas (44,45). In the present studies, we showed that γ -synuclein over-expression activates the survival factor ERK1/2 and blocks the activation of JNK. Activation of JNK can initiate the mitochondria involved caspase apoptosis pathway (30). Therefore, we propose that γ -synuclein, in its oncogenic form (over-expressed) contributes to tumor development by protecting cancer cells under adverse conditions through modulating the ERK and JNK pathways (Fig. 11). In addition, we observed that Taxol- and vinblastine-induced cell death is protected by γ -synuclein indicating that anti-cancer drugs that take effect through activating the JNK/caspase apoptosis pathway may not be effective for cancers with high γ -synuclein protein levels.

The effects of γ -synuclein on ERK1/2 and JNK signaling in ovarian cancer cells appears to be specific for γ -synuclein since over-expression of the α - and β -synucleins in A2780 cells had

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

little or no effect on these MAPKs (Figs. 3 & 5). Even though α - and β -synucleins are expressed in a significant fraction of ovarian tumors (4), we did not observe a discernable phenotype associated with over-expression in cultured cells. The reason for these differences are not readily apparent. However, we have recently observed that the subcellular localization of γ -synuclein in tumor cells may be altered. Previous studies have shown that γ -synuclein is diffusely distributed in the cytoplasm of peripheral neurons, although it is also expressed in the brain (46). We have found in tumor cells which over-express the wild-type γ -synuclein that the protein tends to accumulate in the nuclei (Pan and Godwin, unpublished data). This may also help explain why only a portion of ERK1/2 and JNK co-immunoprecipitates with γ -synuclein (Fig. 2), since the cytoplasmic levels are low in A2780 cells. Furthermore, the amino acid sequences of α - and β -synucleins are more closely related to each other than γ -synuclein (8,9) and the γ -synuclein protein is the least conserved of the synuclein proteins (8,9). Therefore, it is possible that the conserved sequences of α , β , and γ -synuclein may be involved in the interaction with ERK1/2 and that the non-conserved regions (predominantly the C-terminus) may contribute to activation of ERK in a cell type specific manner. Therefore, the effects that we are observing may be both cell type and isoform specific. Additional studies will be needed to better resolve these differences.

Even though we have made some significant observations in this study, the biochemical function of γ -synuclein remains largely unknown. Many of our approaches have come from the study of α -synuclein. α -Synuclein, the most extensively studied synuclein, is the major component of Lewy bodies in sporadic PD, dementia with LBs (DLB), and a subtype of Alzheimer's disease known as the LB variant of Alzheimer's disease (19,47,48). Mutations in

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

the α -synuclein gene have also been linked to familial Parkinson's disease (49-51). Based on homology to 14-3-3 protein and *in vitro* assay, synucleins are hypothesized to be proteins with chaperone properties (20,26). α -Synuclein has been shown to be associated with many of the proteins that interact with 14-3-3 proteins, including PKC, BAD, and ERK (16,21,25). α -Synuclein has also been shown to associate with ERK2 (28) and may reduce the phosphorylation of MAPKs in neurons (29), while synoretin can activate Elk1 pathway when transfected in HEK 293 cells (9). We have demonstrated that in ovarian cancer cells that both γ - and α -synucleins can associate with ERK1/2 (Fig. 2). However, only γ -synuclein but not α -synuclein lead to constitutive activation of ERK indicating that the function of α -synuclein might be different in cancer cells versus neuronal cells. Studies are underway to determine whether this constitutive activation is dependent on the C-terminal sequences which are unique to γ -synuclein.

The second prominent observation we made in this study was that ectopic expression of γ -synuclein resulted in enhanced resistance to the chemotherapeutic drugs, Taxol and vinblastine (Figs. 7-9). In contrast, when these same cells were treated with etoposide, a DNA damage agent, γ -synuclein over-expression did not enhanced cell survival (Fig. 10). There are two main apoptosis pathways initiated from the cell surface membrane, one is initiated by Fas and other death receptors, while the other is initiated by stress signals (30). JNK activation is an essential component of the latter apoptotic signaling (30,52). In addition, many chemotherapy drugs also take effect partly through activating the JNK signaling (34-36). Our data indicate that abnormal over-expression of γ -synuclein might be one of such mechanisms in breast and ovarian cancer that permits tumor cells to overcome the JNK activated apoptotic signaling (30). Both Taxol and vinblastine robustly induced JNK activity in A2780 cells, while the etoposide treatment did little

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

(Figs. 8-10). In γ -synuclein over-expressing cells, this induction by Taxol and vinblastine was suppressed 2- to 3-fold and cell survival was dramatically enhanced. Taken together, these results indicate that the cell death in ovarian cancer cells induced by Taxol, vinblastine, or other drugs that induce JNK, may be protected by γ -synuclein. Furthermore, it is interesting to note that only ~35% of breast tumors and ~50% of all epithelial ovarian tumors possess mutations in the *TP53* gene (53,54). As JNK activation in UV or Taxol treated cells is independent of DNA damage (31,55-57), it is interesting to speculate that over-expression of γ -synuclein may promote tumor cell survival in the presence of p53 wild-type cells. Since γ -synuclein is not expressed in normal breast and ovarian epithelial cells but is expressed in the majority of late-stage breast and ovarian cancers (4,5), it may be a very promising target to develop drugs for the therapies of these diseases.

Our results of γ -synuclein in cancer cells seem different from those of α -synuclein in neuronal cells. It remains to be determined whether these differences are caused by the different cellular context between cancer and neuronal cells, by different subcellular localization of the proteins in various normal and tumor cell types, or by innate difference in protein functions between γ - and α -synucleins. α -Synuclein over-expression in HEK 293 cells or A2780 cells does not induce apoptosis [Iwata, 2001 #101; Pan and Godwin, data not shown]. In neurons, mutant α -synuclein may accelerate apoptosis (58-61) while wild-type α -synuclein may induce or block apoptosis, depending on the path of apoptosis induction (29,58-60,62). In Parkinson's diseases, mitochondrial dysfunction and oxidative stress are believed to be the two main causes for neuronal death (63). The apoptotic role of wild-type α -synuclein in neuronal apoptosis may be secondary by disrupting mitochondria and causing oxidative stress (64). There is evidence

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

that the roles of synuclein in neurodegenerative diseases appear to be quite different. In contrast to the role of α -synuclein in neuronal degeneration, γ -synuclein does not cause neuronal apoptosis (65). γ - and β -Synucleins are not detected in Lewy bodies or Lewy neurites (8,14), and they are intrinsically less fibrillogenic than α -synuclein and cannot form mixed fibrils with α -synuclein (66). Therefore, it would be of interest to determine whether the normal protective role of γ -synuclein is lost in the neurons of PD and other neurodegenerative diseases. Overall, our studies provide new insight into the biological function of γ -synuclein and its role in the pathogenesis of the breast and ovary and offer a new therapeutic target for future treatment.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

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FIGURE LEGENDS

Fig. 1. Establishment of cell lines that ectopically over-express γ -synuclein. Whole cell lysates from the parental cells or stably-transfected cells were analyzed by SDS-PAGE and immunoblotting. Protein levels of γ -synuclein were determined by Western blotting with γ -2, a polyclonal antibody specific for γ -synuclein. Protein loading levels were evaluated by immunoblotting with anti- α -actin antibody. The numbers beneath each band represent the densitometry units (A2780 was assigned the arbitrary unit 1.0 in the α -actin blot). γ -Synuclein protein levels were normalized to the protein levels of α -actin. The graph represents the average \pm S.E. of at least three independent experiments, and representative blots are shown here. Molecular mass standards (in kilodaltons) are indicated to the left of each blot.

Fig. 2. Interaction between γ - and α -synucleins with ERK1/2 and JNK. Cell lysate from A2780, A2780/gam or A2780/alpha were immunoprecipitated with Syn303, NIg (normal IgG) or irrelevant antibodies (not shown) as described in the Experimental Procedures section. The proteins in the immunoprecipitates were identified by immunoblotting with antibodies against ERK1/2, JNK1, p38, and γ -2 (a polyclonal antibody specific for γ -synuclein). Molecular mass standards (in kilodaltons) are indicated on the left. Non-specific bands around the IgG heavy (**) and light (*) chains are indicated by asterisks.

Fig. 3. Activation of ERK in cells over-expressing γ -synuclein. A, ERK1/2 activation is enhanced in γ -synuclein over-expressing A2780 cells. Whole cell lysates (40 μ g/lane) from A2780 cells (parent) or A2780 cells transfected with γ -, β -, or α -synuclein were separated by SDS-PAGE and blotted with appropriate antibodies. The levels of activated ERK or total

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

ERK1/2 were determined using an anti-phospho-ERK specific antibody or ERK1/2 antibodies, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band (the integrated densitometry reading number of A2780 or OVCAR5 was assigned to an arbitrary unit 1.0, and the other readings were normalized thereafter). The synucleins were evaluated by blotting with specific antibodies, i.e., γ -2 for γ -synuclein, Syn207 for β -synuclein, and Syn204 for α -synuclein, respectively. **B**, activation of ERK by γ -synuclein in OVCAR5 cells but not HEK 293 cells. Whole cell lysate (40 μ g/lane) from parental or γ -synuclein over-expressing cells were separated and blotted as in **A**. Panels **A** and **B** are representative of at least three independent experiments with comparable results. **C**, fold increase of ERK activation. The data shown are the average \pm S.E. of three independent experiments. Phosphorylated ERK was normalized to the protein level of total ERK. The basal levels of ERK phosphorylation in the parental A2780 or OVCAR5 cells were set as 1.0. (*) Represents significant difference compared to the parental cells ($p < 0.05$).

Fig. 4. Requirement of MEK1/2 for γ -synuclein enhanced ERK1/2 activation. **A**, A2780 and A2780/gam cells untreated or treated with the MEK1/2 inhibitor, U0126 (10 μ M), were lysed and 30 μ g of proteins were loaded into each lane. As in **Fig. 3**, anti-phospho-ERK1/2 specific antibody was used to detect activated ERK1/2; and the antibody against ERK1/2 was used to detect the protein level of total ERK1/2. **B**, the interaction between ERK and γ -synuclein is independent of the activation status of ERK1/2. A2780/gam cells in normal 10% FBS medium, 10% FBS medium with U0126 (10 μ M), or serum-free medium were lysed and immunoprecipitated with Syn303 or control IgG. The proteins in the immunoprecipitates were detected with the antibodies against ERK1/2 or against human γ -synuclein (γ -2). The

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

autoradiogram shown is the representative of three independent experiments with comparable results.

Fig. 5. Inhibition of JNK activation by γ -synuclein in response to UV treatment. A2780, A2780/gam, A2780/beta, OVCAR5, OVCAR5/gam cells were un-treated or treated with UV (20 J/M²) and cells were lysed at 30 min. JNK activities were analyzed by an immunocomplex kinase assay using GST-c-JUN as substrate. The phosphorylated GST-c-JUN by activated JNK was evaluated by immunoblotting with anti-phospho-c-JUN specific antibody (see Experimental Procedures for details). The protein levels of JNK and γ -synuclein were determined by immunoblotting with anti-JNK and γ -2 antibody, respectively. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of total JNK proteins. The graph above the blots is the average \pm S.E. of three independent experiments. (*) Represents significant inhibition of JNK activation compared to that in the parental cells ($p < 0.05$).

Fig. 6. JNK activation is down-regulated by over-expression of γ -synuclein in response to sodium arsenate. HEK293, OVCAR5, and A2780 cells and their γ -synuclein over-expressing counterparts were treated with Na-arsenite (50 μ M) for 6 hrs. Cells were lysed and the extracts assayed for JNK activity as described in the legends for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

representative of three independent experiments with comparable results.

Fig. 7. γ -Synuclein protects cells from paclitaxel (Taxol) induced cell death and is partially mediated by ERK activation. **A**, Taxol does not affect ERK activity or γ -synuclein mediated ERK activation. A2780 or A2780/gam cells pre-treated with or without U0126 (10 μ M) were treated with Taxol (30 μ M) in the absence or presence of U0126 for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **B**, cell death induced by Taxol was significantly reduced in cells over-expressing γ -synuclein. A2780 and A2780/gam cells treated with Taxol for 48hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining (shown) and WST-1 assays (not shown). The graph represents the average \pm S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$).

Fig. 8. Taxol activated JNK and caspase-3 apoptotic pathway is blocked by γ -synuclein. **A**, down-regulation of JNK activation by Taxol in cells over-expressing γ -synuclein. Cell lysates from A2780 and A2780/gam cells treated with or without Taxol (30 μ M) for 60 min were assayed for JNK activity (see the legend for Fig. 5 for experimental details). The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. JNK activities were measured by levels of phosphorylated c-JUN and normalized to levels of

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

total JNK proteins. The graph above the blots is the average \pm S.E. of three independent experiments. **B**, down-regulation of caspase-3 activity activated by Taxol in A2780/gam cells. A2780 and A2780/gam cells treated with Taxol (30 μ M) for different time lengths were lysed, and approximately 20 μ g protein were incubated with the caspase-3 substrate Ac-DEVD-pNA for 4hr at 37°C. Cleavage of the substrate to release pNA was monitored at 405 nm using a microplate reader. The graph represents the average \pm S.E. of three independent experiments. In panels A and B, (*) represents significant difference compared to that in the parental cells ($p < 0.05$).

Fig. 9. Vinblastine induced cell death and activation of the MAPK pathways in γ -synuclein over-expressing cells. **A**, the cell death induced by vinblastine was significantly reduced in cells over-expressing γ -synuclein. A2780 and A2780/gam cells treated with vinblastine (0.1, 1, or 10 μ M) for 30 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average \pm S.E. of three independent experiments. (*) Represents significant difference in cell death between A2780 and A2780/gam cells ($p < 0.05$). **B**, inhibition of JNK activation by γ -synuclein in response to vinblastine treatment. A2780 and A2780/gam cells were un-treated or treated with vinblastine (1 μ M, and 10 μ M) for 30 min and 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in the legend for Fig. 5. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C**, the effect of vinblastine on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with vinblastine (1 μ M and 10 μ M) in the absence or presence of U0126 (10

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

μ M) for 30 min. Whole cell lysates were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown are representative of three independent experiments with comparable results.

Fig. 10. Effects of γ -synuclein over-expression on etoposide induced cell death and activation

of the MAPK pathways. A, cell death induced by etoposide was not significantly altered in cells that over-express γ -synuclein. A2780 and A2780/gam cells treated with etoposide (1 μ M, 10 μ M, 100 μ M) for 56 hr in the presence or absence of the MEK1/2 inhibitor, U0126. Cell death was determined by trypan blue staining. The graph represents the average \pm S.E. of three independent experiments. **B**, vinblastine treatment does not induce JNK activation. A2780 and A2780/gam cells treated with or without etoposide (10 μ M, and 100 μ M) for 30 min and/or 60 min before lysis for JNK activity assay. JNK activities were analyzed as described in Fig. 5.

The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results. **C**, effect of etoposide on ERK phosphorylation. A2780 or A2780/gam cells pre-treated with or without U0126 were treated with etoposide (10 μ M and 100 μ M) in the absence or presence of U0126 (10 μ M) for 30 min. Whole cell lysate were separated by SDS-PAGE and blotted with phospho-ERK to determine the level of active ERK, or with ERK1/2 antibodies for the level of total ERK1/2. The number beneath each band represents the arbitrary densitometry units of the corresponding band. The blots shown here are representative of three independent experiments with comparable results.

γ -Synuclein promotes cancer cell survival by affecting MAPK pathways

Fig. 11. A proposed model illustrating how γ -synuclein promotes cell survival by modulating the ERK and JNK pathways.

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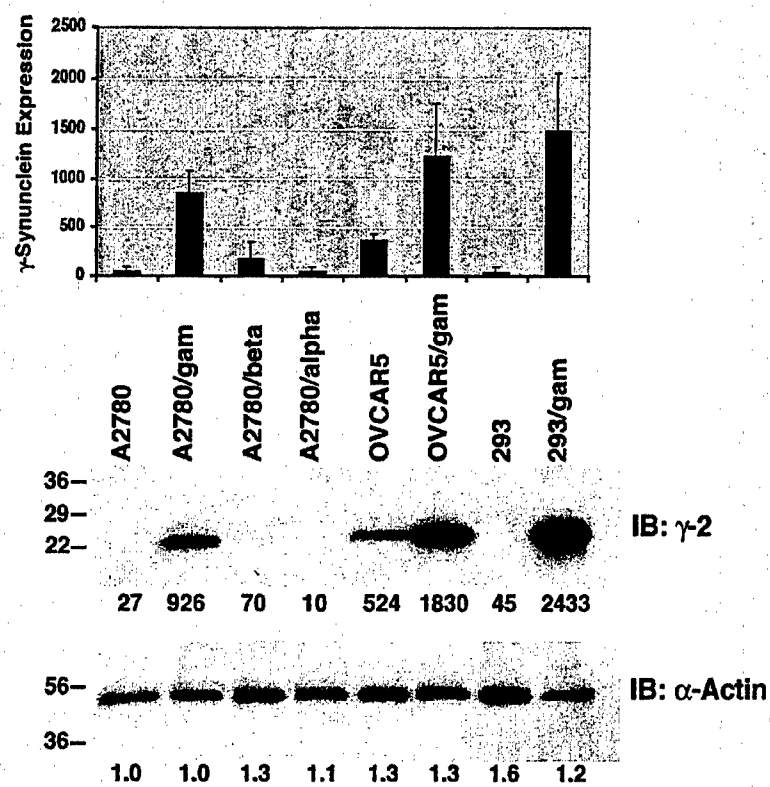
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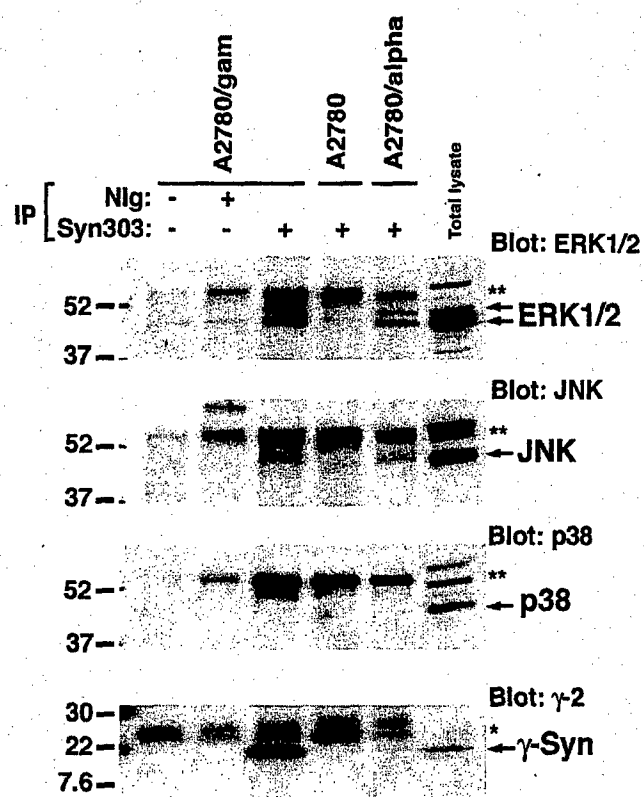
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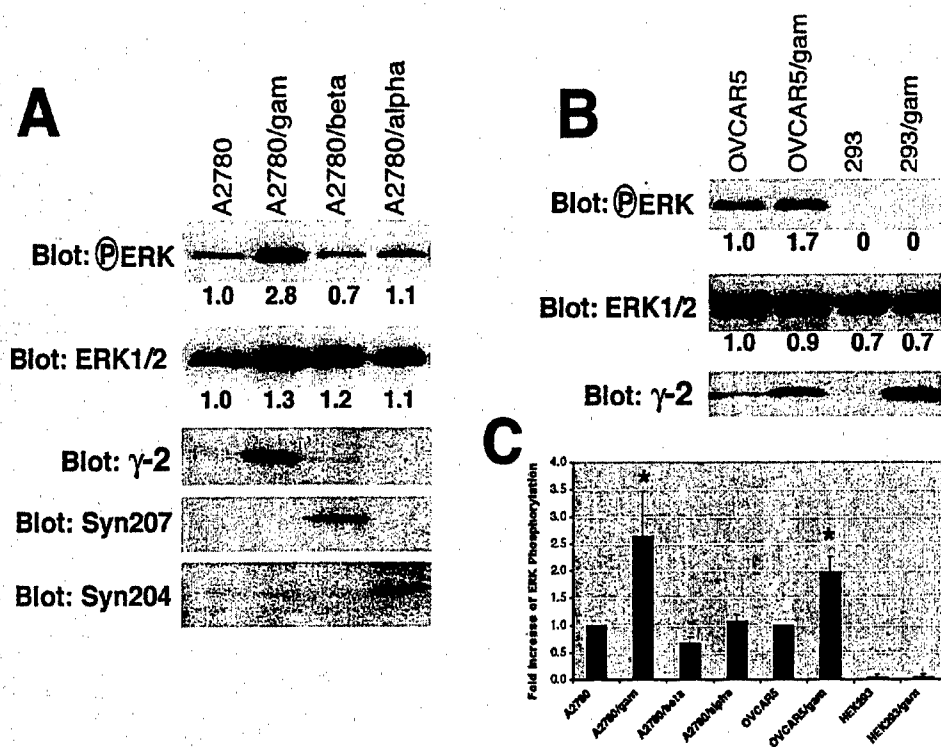
Pan et al. Fig.1



Pan et al. Fig.2

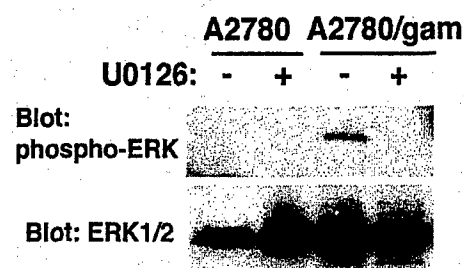


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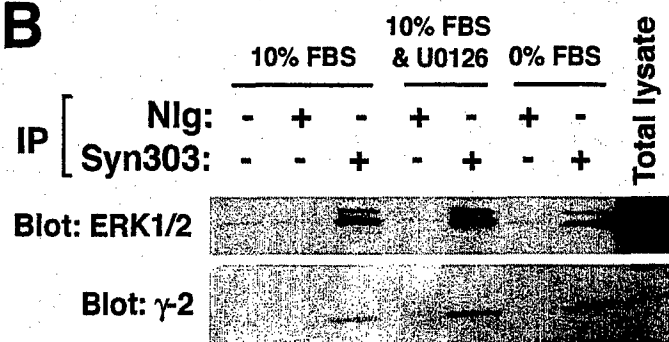


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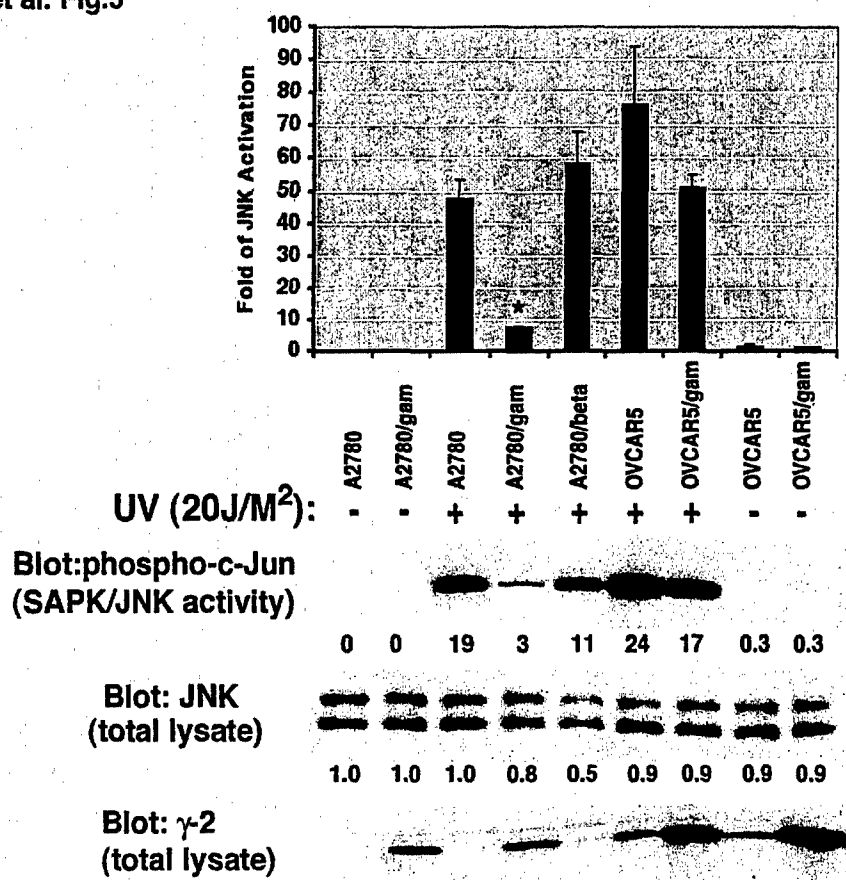
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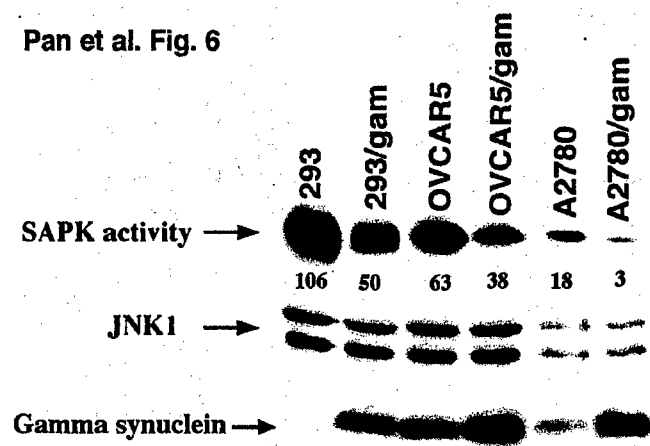
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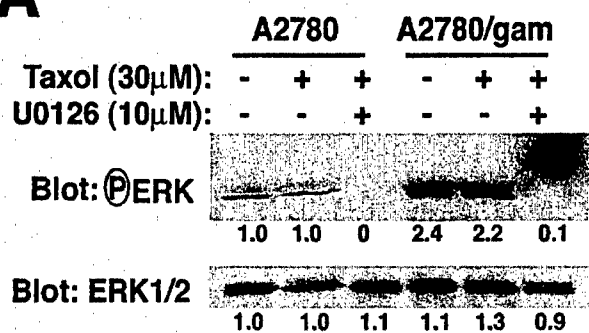


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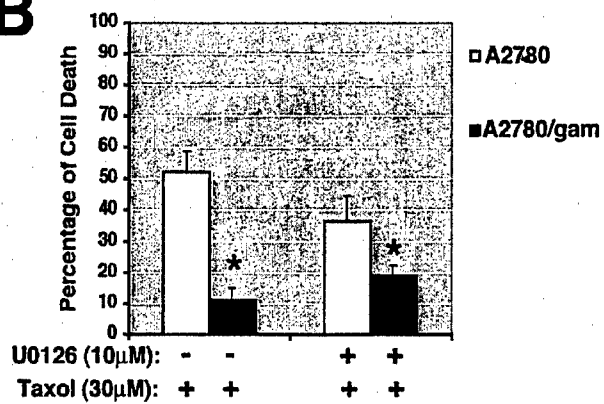


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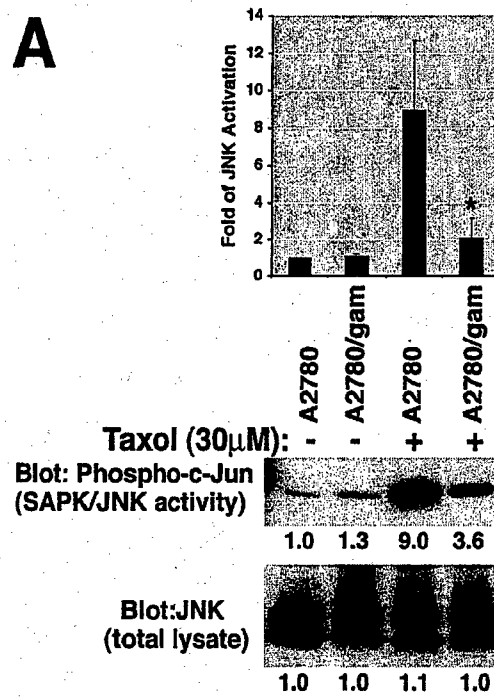


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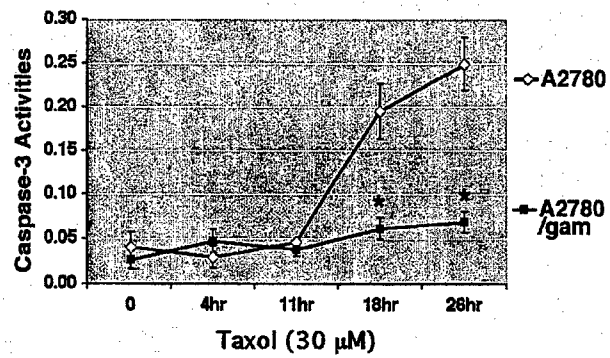


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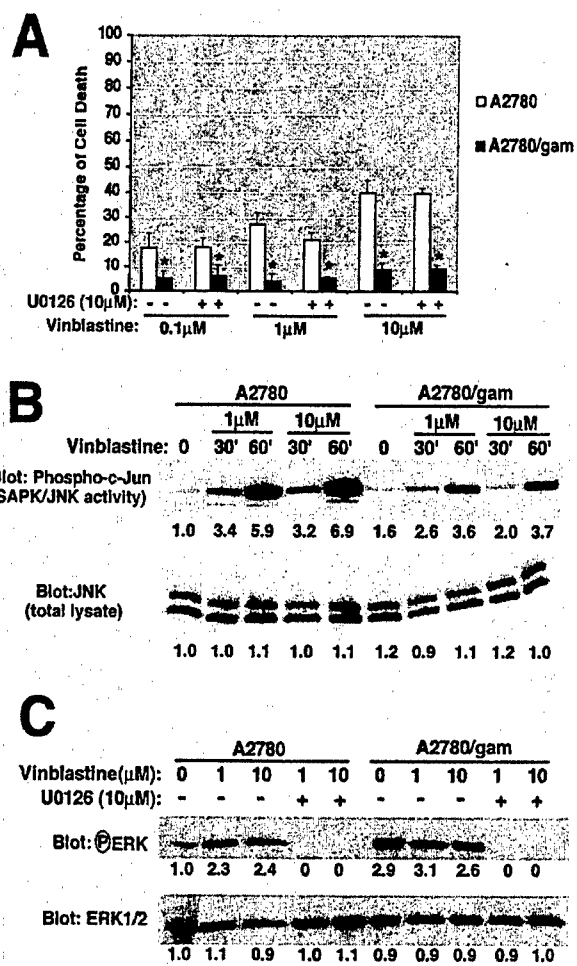
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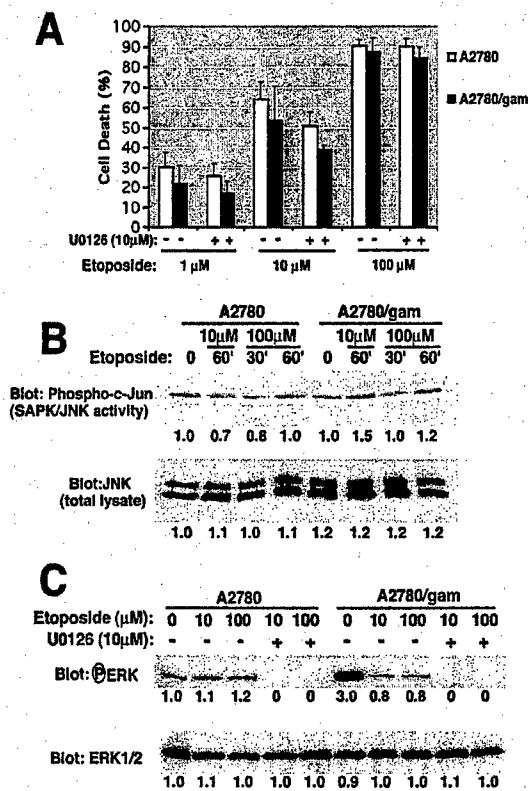
B



Pan et al. Fig.9



Pan et al. Fig.10



Pan et al. Fig.11

